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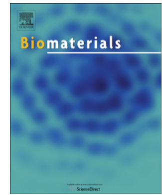

The role of valvular endothelial cell paracrine signaling and matrix elasticity on valvular interstitial cell activation

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ABSTRACT

The effects of valvular endothelial cell (VlvEC) paracrine signaling on VIC phenotype and nodule formation were tested using a co-culture platform with physiologically relevant matrix elasticities and diffusion distance. 100 μm thin poly(ethylene glycol) (PEG) hydrogels of 3–27 kPa Young's moduli were fabricated in transwell inserts. VICs were cultured on the gels, as VIC phenotype is known to change significantly within this range, while VlvECs lined the underside of the membrane. Co-culture with VlvECs significantly reduced VIC activation to the myofibroblast phenotype on all gels with the largest percent decrease on the 3 kPa gels (~70%), while stiffer gels resulted in approximately 20–30% decrease. Additionally, VlvECs significantly reduced αSMA protein expression (~2 fold lower) on both 3 and 27 kPa gels, as well as the number (~2 fold lower) of nodules formed on the 27 kPa gels. Effects of VlvECs were prevented when nitric oxide (NO) release was inhibited with L-NAME, suggesting that VlvEC produced NO inhibits VIC activation. Withdrawal of L-NAME after 3, 5, and 7 days with restoration of VlvEC NO production for 2 additional days led to a partial reversal of VIC activation (~25% decrease). A potential mechanism by which VlvEC produced NO reduced VIC activation was studied by inhibiting initial and mid-stage cGMP pathway molecules. Inhibition of soluble guanylyl cyclase (sGC) with ODQ or protein kinase G (PKG) with RBrcGMP or stimulation of Rho kinase (ROCK) with LPA, abolished VlvEC effects on VIC activation. This work contributes substantially to the understanding of the valve endothelium's role in preventing VIC functions associated with aortic valve stenosis initiation and progression.

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1. Introduction

Fibrocalcific aortic valve disease (FCAVD) is typically classified by fibrosis and calcification of the aortic heart valve. This disease affects a surprisingly large portion of the US population, with 2–4% of adults over the age of 65 diagnosed with FCAVD and 70,000 aortic valve replacement surgeries occurring annually [1–3]. While FCAVD was initially thought to result from a progressive wearing out of the valve tissue over time, it is now understood to be a dynamic process, which involves resident valvular interstitial cells (VICs) [1]. VICs are critical in maintaining aortic valve homeostasis and function through many actions, such as proliferation [4–6], secretion of matrix metalloproteinases (MMPs) [4,5,7,8], and *de novo* extracellular matrix (ECM) molecules [4–6,9–11]. However, when inflammatory signaling is prolonged with repeated valve injury, as in hypertension or diabetes [12,13], regulation of VIC phenotype can be lost. If myofibroblastic VICs persist, VICs can increase valve stiffness through excess remodeling of the valve tissue (e.g., excess collagen deposition), and cause VICs to express genes associated with osteogenesis [14,15]. This process of pathological VIC activation to secretory myofibroblasts and eventually osteoblast-like cells is facilitated by several factors, including the potent cytokine transforming growth factor-β1 (TGF-β1) [4,7]. Unfortunately, very few cues are known to prevent VIC activation, like fibroblast growth factor (FGF) [16] and no small molecule or drug has been found that reverses VIC activation *in vivo* or *in vitro,* once it has occurred.

VICs reside in the interstitium of the valve tissue, and this tissue is lined with valvular endothelial cells (VlvECs) [17,18], that provide a protective and selective barrier between blood and VICs [19,20]. Interestingly, initiation of aortic sclerosis (i.e., an early stage of FCAVD) has been linked to endothelial cell dysfunction [17,21]. Endothelial cells appear to be a critical modulator of the

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progression of CAVD by regulation of VIC activation and calcification [17,22–25]. This regulation of VIC phenotype has been correlated with VIvEC produced nitric oxide (NO), as synthetically generated NO can significantly decrease calcific nodule formation in VIC cultures on treated tissue culture plastic [26]. But tissue culture plastic has an elastic modulus of ~1 GPa, which is known to cause high levels of VIC myofibroblast activation [14]. Co-culture of VICs with vascular endothelial cells (VECs) isolated from the aorta, or addition of synthetically generated NO, also decrease activation of VICs cultured in osteogenic media (supplemented with dexamethasone) [24]. However, endothelial cells likely secrete multiple beneficial signaling factors (e.g., fibroblast growth factor (FGF) [27] and prostaglandins [28]), which might also suppress VIC activation.

Previous studies have yielded valuable insight into potential signaling pathways associated with endothelial regulation of VIC phenotype, although activation of VICs has been induced primarily with biochemical factors (e.g., dexamethasone). Additionally, less is known about the role of microenvironmental elasticities, which is an important aspect of valve disease progression toward FCAVD (i.e., tissue stiffening). Thus, we sought to use a simple co-culture system that would allow one to study the role of microenvironmental mechanical cues on VIC activation in the presence of VIvECs. We tested the hypothesis that matrix elasticity may modulate the interaction of VICs and VIvECs, as the ability of biochemical cues to reverse or inhibit VIC activation may vary with the local microenvironmental stiffness.

Culture substrate elasticity has been shown to direct the activation of VICs to myofibroblasts [10,15,29]. Specifically, Kloxin et al. demonstrated that culturing VICs on a substrate with a Young's modulus (E) of 33 kPa, VICs differentiated into a predominately myofibroblastic population (~80%) [29]. Conversely, when VICs were cultured on a softer substrate (E~7 kPa), the population was mainly fibroblastic. Clearly, biophysical cues are an important aspect of VIC phenotype regulation, and having adept control of the culture substrate elasticity provides a useful tool for studying the role of mechanical signals in FCAVD initiation and progression. We have used peptide-functionalized poly(ethylene glycol) (PEG) hydrogels as a VIC culture platform that facilitates control of both biophysical and biochemical microenvironmental cues [9,14,29–31]. Part of the motivation for using PEG-based materials relates to their high water content (similar to that of many soft tissues), minimal nonspecific adsorption of protein, and mechanical moduli of physiological relevance to valve tissue [10,32,33].

In this study, VICs were cultured on ~100 μm thin PEG hydrogels to allow for a physiologically relevant diffusion distance between VIvECs and VICs, while the crosslinking density of the gels was controlled to yield materials of varying Young's moduli. This range of elasticity was selected to direct VIC activation from mostly quiescent fibroblasts to activated myofibroblasts, allowing control of the percentage of myofibroblasts in the VIC population, which increases during valve tissue stiffening associated with FCAVD progression [34,35]. Then, the effect of VIvECs on VIC phenotype and nodule formation were assessed by lining the underside of the insert membrane with VIvECs and analyzing αSMA immunostaining or bright field images of formed nodules, respectively. The key paracrine signaling molecule and its mechanism were studied with small molecule inhibitors and assessment of VIC activation.

2. Materials and methods

2.1. Materials

Eight-armed poly(ethylene glycol) (PEG, M_n: 20 & 40 kDa) was purchased from JenKem. All amino acids and resin for solid phase peptide synthesis were purchased from Chem-Impex and Novabiochem, respectively. Porcine hearts were obtained from Hormel Inc. for VIC and VIvEC isolation, and M199 and porcine endothelial media were purchased from Life Technologies and Genlantis, respectively. All other chemicals were purchased from Sigma–Aldrich, unless otherwise specified.

2.2. Monomer synthesis

Eight-armed PEG-norbornene (PEG-N) (M_n: 20 & 40 kDa) was synthesized as previously described by Fairbanks et al. [32]. Briefly, the reaction was carried out under anhydrous conditions in the organic solvent dichloromethane (DCM), where a PEG solution was added drop-wise to a stirred solution of N,N′-dicyclohexylcarbodiimide (DCC) and norbornene acid, and allowed to react overnight at room temperature. The norbornene functionalized PEG in this solution was then precipitated in ice-cold ethyl ether, filtered, and re-dissolved in chloroform. This chloroform PEG solution was then washed with a glycine buffer and brine before being precipitated in ice-cold ethyl ether and filtered again. The filtered PEG was then placed in a vacuum chamber to remove excess ether. The percent functionalization of PEG arms with norbornene groups was determined using 1H NMR by comparing the hydrogen peaks associated with the carbon adjacent to the ester linkage (~4.2 ppm) to the hydrogen peaks associated with the PEG molecule (~3.6 ppm). Only synthesis products with greater than 95% functionalization were used in subsequent experiments.

Two peptides were included in the thiol-ene formulation, one derived from fibronectin (CGRGDS) [36] to promote cell adhesion and a non-degradable dithiol linker peptide (KCGGPQGI$_d$WGQGCK) [32,37], to facilitate crosslinking of the polymer network, where d denotes a reversed d chirality of the I amino acid. These peptides were synthesized using solid phase peptide synthesis (SPPS) on a Protein Technologies Tribute peptide synthesizer. After a 5 wt% phenol trifluoroacetic acid (TFA) cleavage and ice-cold ether precipitation, if the purity was found to be less than 95% via high pressure liquid chromatography (HPLC), then large scale HPLC purification was performed. The correct eluate fraction, based on MW, was determined through matrix assisted laser desorption ionization (MALDI). The HPLC buffer was removed from the peptide in solution via lyophilization.

2.3. Hydrogel fabrication

In a sterile cell culture hood, eight-armed PEG-N, non-degradable dithiol linker peptide, CGRGDS, and 0.2 mM lithium phenyl-2,4,6-trimethylbenzoylphosphinate (LAP) [38] were dissolved in sterile PBS to yield the final monomer solution concentrations indicated in Table 1. As drawn schematically in Fig. 2a, these monomer solutions were transferred onto a Teflon pedestal, with enough volume (10 μl) to create a ~100 μm thin gel (Fig. 2b and c), and a 12-well insert (0.4 μm pore size) was then pressed on top. The Teflon pedestal, monomer solution, and insert sandwich were then placed under 365 nm UV light at 3 mW/cm^2 for 2 min [38]. The resulting gels were allowed to swell overnight in serum free, low glucose DMEM media at 37 °C before cell seeding.

For formed gel thickness characterization, a fluorescent CGRGDS peptide was covalently tethered in the transwell gel and allowed to swell overnight in PBS. The gel was then imaged on a Zeiss 2-photon confocal microscope, taking a z-stack in 10 μm increments from 50 μm below the membrane to 150 μm above (Fig. 2b). Significant fluorescence persisted from the membrane to approximately 105 μm above (i.e., the actual gel thickness) (Fig. 2c).

Final gel Young's modulus (E) was determined by measuring the shear modulus (G) of swollen hydrogels via parallel plate rheometry and converting to Young's modulus via rubber elasticity theory ($E = 2^*(1+\nu)^*G = 3^*G$), which assumes a Poisson's ratio (ν) of 0.5 for an incompressible material (water) [32].

2.4. VIC and VIvEC isolation and culture

Primary VICs and VIvECs were isolated from aortic leaflets, which were excised from fresh porcine hearts acquired from Hormel within 24 h of slaughter via a sequential collagenase digestion as previously described [39,40], and aliquots were frozen until needed. Briefly, the leaflets were incubated in Earle's balanced salt solution containing 250 U/mL collagenase for 10 min to remove the VIvECs, followed by an additional 20 min of collagenase digestion to remove the remaining endothelium. VICs were then removed by continuing incubation in fresh collagenase solution for 60 min.

The VIvEC suspension was pelleted and resuspended in porcine endothelial media supplemented with 15 v/v% fetal bovine serum (FBS), 100 U/mL penicillin, 100 μg/mL streptomycin, and 1 μg/mL fungizone; plated on TCPS dishes; and cultured to 100% confluency at 37 °C and 5% CO_2. The VIvECs were then trypsinized, pelleted, and resuspended in 45 v/v% porcine endothelial media, 50 v/v% FBS, and

Table 1
Monomer concentrations and PEG-N molecular weights for each of the final Young's modulus materials used for cell culture.

PEG-N M_n (kDa)	PEG-N (mM)	Thiol to Ene ratio	Dithiol linker peptide (mM)	CGRGDS (mM)	Young's modulus (kPa)
40	2.5	2 to 3	6.0	2.0	3
40	2.5	1 to 1	9.0	2.0	10
20	5.0	3 to 4	14.3	2.0	15
20	5.0	1 to 1	19.0	2.0	27

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